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(54) HLA typing method and cDNA probes used therein.

(57) HLA typing based on restriction length polymorphism is carried out by: digesting an individual's HLA DNA with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA; subjecting the digest to genomic blotting using a labeled cDNA hybridization probe that is complementary to an HLA DNA sequence involved in the polymorphism; and comparing the resulting genomic blotting pattern with a standard. This technique may be adapted to make paternity or transplant or transfusion compatibility determinations or to make disease association correlations to diagnose diseases or predict susceptibility to diseases. Locus specific cDNA hybridization probes, particularly probes for genes of Class II loci (D and DR loci), for use in the typing procedure are described.

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HLA TYPING METHOD AND  
cDNA PROBES USED THEREIN

Description

Technical Field

5           The invention is in the fields of genetic  
engineering and human genotyping. More specifically  
the invention concerns methods for HLA typing based on  
HLA DNA restriction fragment length polymorphism and  
to novel complementary DNA (cDNA) probes that are used  
10 in such methods.

Background Art

          The major histocompatibility complex (MHC)  
of humans is a cluster of genes occupying a region  
located on the sixth chromosome. This complex,  
15 denoted HLA (Human Leukocyte Antigen), has been  
divided into five major gene loci, which according to  
World Health Organization nomenclature are designated  
HLA-A, HLA-B, HLA-C, HLA-D, and HLA-DR. The A, B, and  
C loci are single gene loci. The D and DR loci are  
20 multi-gene loci. The A, B, and C loci encode the  
classical transplantation antigens, whereas the D and  
DR loci encode products that control immune respon-  
siveness. More recent definitions divide the gene  
products of the HLA loci into three classes (I, II,  
25 and III) based on structure and function (Nature  
(1982) 297:692-632). Class I encompasses the products  
of the HLA-A, HLA-B, and HLA-C loci and the Qa/TL  
region. The products of the HLA-D and HLA-DR related  
genes fall in Class II. The Class II antigens are  
30 believed to be heterodimers composed of an  $\alpha$  (~34,000  
daltons) glycopeptide and a  $\beta$  (~29,000 daltons) glyco-

peptide. The number of loci and the gene order of Class II are tentative. Class II currently includes loci designated DR $\alpha$ , DR $\beta$ , DS $\beta$ , DC( $\alpha$  or  $\beta$ ), and SB. It is likely that future investigation will reveal additional Class II loci. The third class, Class III, includes components of complement. As used herein, the term "HLA" is intended to include the above described loci as well as loci that are closely linked thereto.

10           The products encoded by the HLA loci are currently typed serologically or by mixed lymphocyte culture methods. Such typing is used in paternity determinations, transplant and transfusion compatibility testing, blood component therapy, anthropological studies and in disease association correlation to  
15           diagnose diseases or predict susceptibility to diseases. The major drawbacks to such HLA typing, particularly of the Class II loci, are the complexity of the sera and the lack of widespread availability of  
20           standard sera necessary to conduct the tests. Also, since serological typing is based on reactions of sera with the HLA gene products it may not be useful for fetal HLA typing in the early stages of pregnancy when those products have not yet been expressed. Further,  
25           the lymphocytotoxicity test often gives results that do not provide an adequate basis for recognizing Class II locus specificities.

          It is well known that there is extensive polymorphism in the DNA of the human population.  
30           Recent work has also found polymorphism in the restriction endonuclease digests of human DNA. Restriction endonucleases recognize specific nucleotide sequences in DNA and catalyze endonucleolytic cleavages, yielding DNA fragments of defined length. Dif-

ferences among individuals in the lengths of a particular restriction fragment are called "restriction length fragment polymorphisms" (RFLPs). Kan and Dozy, PNAS (1978) 75:5631-5635 report RFLPs produced by HpaI cleavage of human  $\beta$ -globin genes and an apparent association between a 13.0 kb variant of the normal 7.6 kb fragment and sickle hemoglobin mutation. These RFLPs were detected by comparing Southern blots of HpaI restricted cellular DNA from individuals with normal hemoglobin, sickle cell trait, and sickle cell anemia probed with a radiolabeled  $\beta$ -globin cDNA probe.

Botstein, et al, Am J Human Genet (1980) 32:314-331, have proposed using RFLPs as genetic markers to construct a genetic linkage map of the human genome. Their proposal contemplates identifying polymorphic loci by Southern blotting using restricted DNA and single strand cDNA probes, testing the loci for linkage relationships in human pedigrees, and arranging the loci into linkage groups to form a genetic map.

Sood, et al, PNAS (1981) 78:616-620 (also PCT application 8202060 published 24 June 1982), describe the isolation of cDNA clones for HLA-B antigens. These clones were prepared by synthesizing cDNA from an mRNA mix containing mRNA coding for the desired HLA antigen, inserting the cDNA into a vector, transforming a bacterial host and isolating transformant clones that contain the desired DNA segment by probing with an oligonucleotide probe that is specific for the desired DNA sequence. Ploegh, et al PNAS (1980) 77:6081-6085 have also reported cloning a cDNA probe for an HLA gene sequence.

Disclosure of the Invention

One aspect of the invention is an HLA typing method based on HLA restriction length polymorphism characterized by the following steps:

5 (a) digesting HLA DNA from an individual with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA;

(b) subjecting the digest of (a) to genomic blotting using a labeled cDNA hybridization probe that  
10 is complementary to an HLA DNA sequence involved in the polymorphism; and

(c) comparing the genomic blotting pattern obtained in (b) with a standard genomic blotting pattern for said HLA DNA sequence obtained using said  
15 restriction endonuclease and an equivalent labeled cDNA hybridization probe.

As used in connection with describing the probe the term "equivalent" is intended to mean the same probe or one having the same specificity.

20 Another aspect of the invention is an alternative embodiment of the above described method in which the blotting step is replaced by a solution hybridization of the digest with the probe followed by resolution of the hybridize. This alternative  
25 method is characterized by the following steps:

(a) digesting HLA DNA from an individual with a restriction endonuclease that produces a polymorphic pattern with HLA DNA;

(b) solution hybridizing the digest of step  
30 (a) with a labeled cDNA probe that is complementary to an HLA DNA sequence involved in the polymorphism;

(c) resolving the hybridize of step (b);

(d) detecting labeled hybrids in the resolved hybridize; and

(e) comparing the resolution pattern of the labeled hybrids with a standard resolution pattern for said HLA DNA sequence obtained using the restriction endonuclease and an equivalent labeled cDNA probe.

5 As applied to paternity testing the method involves:

(a) digesting HLA DNA of the mother of the individual, the suspected father of the individual, and the individual with a restriction endonuclease  
10 that produces a polymorphic digestion pattern for HLA DNA;

(b) subjecting each of the digests of (a) to genomic blotting (or solution hybridization - resolution) using a labeled cDNA hybridization probe that is  
15 complementary to an HLA DNA sequence involved in the polymorphism; and

(c) comparing the genomic blotting (or resolution) patterns obtained in (b) to determine correspondence between the individual's pattern and the  
20 mother's and suspected father's pattern and thereby determining whether the suspected father is the actual father of the individual.

As applied to determining whether an individual has or is susceptible to a disease the method is  
25 characterized by the following steps:

(a) digesting HLA DNA from the individual with a restriction endonuclease that produces a polymorphic digestion pattern of an HLA locus that is associated with the disease;

30 (b) subjecting the digest of (a) to genomic blotting (or solution hybridization - resolution) using a labeled cDNA probe that is complementary to the DNA sequence of said HLA locus;

(c) comparing the genomic blotting (or resolution) pattern obtained in (b) with a standard pattern of an individual having said disease obtained using said restriction endonuclease and an equivalent  
5 labeled cDNA probe.

As applied to determining transplant or transfusion compatibility the method is characterized by the following steps:

(a) digesting HLA DNA of the transplant or  
10 transfusion donor and the transplant or transfusion host with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA;

(b) subjecting each of the digests of (a) to genomic blotting (or solution hybridization - resolution) using a labeled cDNA probe that is complementary  
15 to an HLA DNA sequence involved in the polymorphism;  
and

(c) comparing the genomic blotting (or resolution) patterns obtained in (b) to determine correspondence therebetween and thereby determining whether  
20 the transplant or transfusion donor's HLA is compatible with the transplant or transfusion host's HLA.

Another aspect of the invention is an HLA cDNA probe characterized in that the probe is specific  
25 to a single HLA locus and comprises a labeled DNA sequence that is complementary to the DNA sequence at said locus.

Another aspect of the invention is an HLA cDNA sequence characterized in that the sequence is  
30 complementary to a single HLA locus.

Brief Description of the Drawings

In the drawings:

Fig 1 is the autoradiograph described in Example 4;

5 Fig 2 is the autoradiograph described in Example 6;

Fig 3 is a restriction map of the HLA-DR $\alpha$  locus showing the location of polymorphic restriction sites for the enzymes BglIII and EcoRV detected with  
10 the probe of Example 1;

Fig 4 is the autoradiograph described in Example 7;

Figs 5, 6, and 7 are the autoradiographs described in Example 8; and

15 Figs 8 and 9 are the autoradiographs described in Example 9.

Modes for Carrying Out the Invention

The initial step in typing an individual's HLA by the invention method is to obtain a sample of  
20 the individual's HLA DNA. As used herein, the term "individual" is intended to include beings that are in a fetal stage. All nucleated cells contain HLA DNA and, therefore, are potential sources for the required DNA. For convenience peripheral blood cells will typ-  
25 ically be used rather than tissue samples. As little as 10 to 100 cc of peripheral blood provide sufficient HLA DNA for typing. In the case of fetal HLA typing, placental cells or amniotic fluid may be used. The DNA is isolated from nucleated cells under conditions  
30 that preclude DNA degradation. Such isolation involves digesting the cells with a protease that does not attack DNA at a temperature and pH that reduces the likelihood of DNase activity followed by extrac-



tion of the digest with a DNA solvent. DNA isolation from nucleated cells is described by Kan, et al, N Eng J Med (1977) 297:1080-1084 and Nature (1974) 251:392-393, and Kan and Dozy, supra. The extracted  
5 DNA may be purified by dialysis, chromatography, or other known methods for purifying polynucleotides.

In the second step of the method the isolated DNA is restricted with a restriction endonuclease that cleaves or cuts DNA hydrolytically at a specific  
10 nucleotide sequence. Sequences so recognized by the enzymes are called restriction sites. Restriction endonucleases that recognize and cleave at specific sites are sometimes referred to as class II restriction enzymes (class I enzymes cleave randomly rather  
15 than at specific sites). Enzymes that produce blunt end DNA fragments (hydrolysis of the phosphodiester bonds on both DNA strands occur at the same site) as well as enzymes that produce sticky ended fragments (the hydrolysis sites on the strands are separated by  
20 a few nucleotides from each other) may be used. In any event, it is essential that the restriction endonuclease be one that produces a polymorphic digestion pattern associated with the HLA locus or loci under investigation. Determinations of which enzymes pro-  
25 duce RFLPs at which loci may be made experimentally using various enzymes in conjunction with various specific HLA cDNA probes in the invention method. Table 1 lists the RFLPs that have been identified to date in this manner.

Table 1

<u>HLA cDNA probe</u>	<u>Enzymes revealing RFLP</u>
pHLA-Dp34 (DR $\alpha$ )	<u>BglII</u> , <u>EcoRV</u>
p29G8 (DR $\alpha$ - related)	<u>BglII</u>
5 pHLA-B7	<u>EcoRI</u> , <u>PvuII</u> , <u>KpnI</u> , <u>XbaI</u> , <u>HindIII</u> , <u>BamHI</u>
pHLA-DR $\beta$ -4	<u>EcoRI</u> , <u>BglII</u>
pHLA-DR $\beta$ -8 (DR $\beta$ - related)	<u>EcoRI</u> , <u>BglII</u>

The digestion of the DNA with the endonuclease may be carried out in an aqueous medium under conditions favoring endonuclease activity. Typically the medium is buffered to a pH of 6.5 to 8.0. Mild temperatures, 20°C to 45°C, preferably physiological temperatures, are employed. Restriction endonucleases normally require magnesium ions and, in some instances cofactors (ATP and S-adenosyl methionine) or other agents for their activity. Therefore, a source of such ions, for instance inorganic magnesium salts, and other agents, when required, will be present in the medium. The amount of DNA in the digestion mixture will typically be in the range of 1% to 20% by weight. In most instances 5 to 20  $\mu$ g of total cell DNA digested to completion provides an adequate sample for typing. Excess endonuclease, usually one to five units/ $\mu$ g DNA, will be used. If desired the restriction digest may be worked up by precipitation and resuspension as described by Kan and Dozy, supra, prior to being subjected to genomic blotting or solution hybridization, as the case may be.

The third step of the process is analyzing the restriction digest by genomic blotting or solution

hybridization and resolution for the presence of one or more HLA gene sequences. In the case of typing for a particular HLA gene the analysis is directed to detecting a DNA sequence that uniquely characterizes that gene. However, when the invention is used for paternity testing or transplant or transfusion compatibility the analysis does not necessarily involve identifying a specific locus or loci but may be done by comparing single or multilocus patterns of one individual with that of another individual using the same restriction endonuclease and an equivalent probe to determine similarities and differences between the patterns. In this regard a single locus probe will identify RFLPs associated with a single HLA locus whereas a multilocus probe will identify RFLPs associated with two or more HLA loci. Three basic steps are involved in the analysis: (1) resolving or separating the fragments by size; (2) annealing the fragments with a labeled cDNA probe that is complementary to the desired HLA DNA sequence(s); and (3) detecting the presence of labeled hybrids. The first two steps in the sequence--separating and annealing--are reversed in the solution hybridization embodiment. The genomic blotting embodiment uses the indicated sequence. The analysis method known as "Southern blotting" that is described by Southern, E.M. (1975) J Mol Biol 98:503-517 is currently a preferred analysis method. In Southern blotting the digestion products are electrophoresed, transferred and affixed to a support which binds nucleic acid, and hybridized with an appropriate labeled cDNA probe. Labeled hybrids are detected by autoradiography.

Electrophoresis is the separation of the digestion products contained in a supporting medium by

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size under the influence of an applied electric field. Gel sheets or slabs, eg agarose or agarose-acrylamide, are typically used as the supporting medium in Southern blotting. The electrophoresis conditions are such as to effect the desired degree of resolution of the fragments. A degree of resolution that separates fragments that differ in size from one another by as little as 100 base pairs will usually be sufficient. Size markers are run on the same gel to permit estimation of the size of the restriction fragments. In carrying out the electrophoresis, the digestion products are loaded onto one end of the gel slab (commonly called the "origin") and the fragments separate by electrically facilitated transport through the gel, with the shortest fragment electrophoresing from the origin towards the other (anode) end of the slab fastest.

After electrophoresis the gel is readied for annealing by placing it in a DNA denaturing solution, conveniently a mild base, generally about 0.2 to 1 M hydroxide, preferably 0.5 M NaOH, to dissociate the DNA strands. After denaturation, the gel is placed in a neutralizing solution and neutralized to a mildly acid pH. The DNA is then transferred to the substrate, which is typically made from materials such as nitrocellulose paper or diazobenzyloxymethyl paper, by contacting the gel with the paper in the presence of reagents, eg buffer, and under conditions, eg light weight and 0°C to 25°C, that promote transfer and covalent or noncovalent binding of the DNA (particularly guanosine and uridine bases) to the sheets. Such reagents and conditions are described by Southern, E.M. *supra*, Wahl, et al, PNAS (1979) 76:3683-3687, Kan and Dozy, *supra*, and US 4,302,204.

After the transfer is complete the paper is separated from the gel and is dried. Hybridization (annealing) of the resolved single strand DNA on the paper to an HLA cDNA probe is effected by incubating the paper with the probe under hybridizing conditions. The hybridization will typically be conducted in an aqueous buffer solution containing a polar solvent such as formamide. Other additives that enhance the hybridization such as sodium chloride, sodium citrate, serum albumin and sonicated denatured DNA such as denatured salmon sperm DNA may be included in the hybridization medium. See Southern, supra, Kan and Dozy, supra and US Pat No 4,302,204, col 5, line 8 et seq.

Complementary DNA probes that are specific to one (locus specific) or more (multilocus) particular HLA DNA sequences involved in the polymorphism are essential components of the hybridization step of the typing method. Locus specific cDNA probes may be made by identifying desired HLA cDNA clones in cDNA libraries constructed from membrane-bound mRNA using synthetic oligonucleotide probes that hybridize to specific HLA cDNA clones. The clones are made detectable by labeling them with a detectable atom, radical or ligand using known labeling techniques. Radiolabels, preferably  $^{32}\text{P}$ , will typically be used. The identified clones may be labeled with  $^{32}\text{P}$  by nick translation with an  $\alpha$ - $^{32}\text{P}$ -dNTP (Rigby, et al, J Mol Biol (1977) 113:237) or other available procedures to make the locus specific probes for use in the invention methods.

The base sequences for the synthetic oligonucleotide probes used to screen the cDNA libraries are determined from HLA antigen amino acid sequences using the genetic code. The amino acid sequences may

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be determined experimentally or from published data eg, Ploegh, et al, supra and Sood, et al, supra. Amino acids with minimal codon degeneracy are used whenever possible. If the amino acid sequence suggests more than one possible oligonucleotide sequence, all possible oligonucleotide sequences that code for the amino acid sequence are made and tested to determine which results in the best probe. Oligonucleotides having the desired base sequences may be prepared using the known phosphate diester, phosphate triester, or phosphite triester techniques. The phosphate triester method described by Good, et al, Nucl Acid Res (1977) 4:2157, is preferred. Blocked nucleotide reagents used in this method are available commercially. The specificity of a synthetic oligonucleotide may be determined by primer extension analysis or by using it as a hybridization probe in "Northern" blot analysis (a technique analogous to the Southern blot method for analyzing mRNA instead of DNA that was developed by Alwine, et al, PNAS (1977) 74:5350) of poly(A<sup>+</sup>) mRNA from a B cell line. Potential locus specific probes may also be identified by hybridizing cDNA library clones with multilocus probes and determining the specificity of the clones that anneal with the multilocus probes.

As more information about the DNA sequences of HLA genes becomes available it will be possible to discern whether there are any sequences that are unique to a particular locus, or perhaps a particular allele. Such information will permit the synthesis of locus specific probes that hybridize only to the unique portion of the gene. In this regard preliminary data have been developed indicating that in the case of the HLA-A and HLA-B loci the distinguishing

portions of the genes may lie in the 3' untranslated regions.

The final step in the method is identifying labeled hybrids on the paper (or gel in the solution hybridization embodiment). Autoradiography is currently used to detect radiolabel-containing hybrids. It involves laying the paper on a piece of radiation-sensitive film (X-ray film). The disintegration of the label results in the deposition of a silver grain in the film. The film is developed and the pattern of labeled fragments is identified. The specificity of the probe and the particular restriction endonuclease used will determine the number of fragments that appear in the pattern. Locus specific probes will typically give patterns with fewer bands than the patterns produced using multilocus probes.

As indicated previously these autoradiographs are used to determine HLA type or, in the case of paternity testing, transplant or transfusion compatibility, and disease association to determine similarities in the autoradiograph patterns of different individuals or similarities between an individual's pattern and a standard pattern, as the case may be. In this regard it will be appreciated that paternity testing and transplant or transfusion compatibility may also be carried out by HLA typing the individuals by the invention method and comparing their HLA types. In HLA typing the fragment(s) appearing on the test autoradiograph is/are compared to the fragment(s) that characterize a particular HLA type to determine correspondence therebetween and thus whether the test subject is that HLA type. This may be done by matching the test autoradiograph with a standard autoradiograph or simply matching the size distribution of the

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fragment(s) appearing on the test autoradiograph with the size distribution of fragment(s) for the standard. By evaluating the HLA DNA RFLP patterns for individuals of known (by conventional HLA typing) HLA type it is possible to assign specific restriction fragments to a given HLA locus. It is also expected that practice of the invention method will identify hitherto unidentified HLA genes, particularly in Class II. In this manner correlations between restriction fragment patterns and HLA type may and will be made. Such correlations may be used in deciphering test autoradiographs. The method also provides a technique for defining subdivisions of serologically defined HLA types. The use of HLA types in paternity tests, transplantation or transfusion testing and in disease diagnosis and prognosis is described in Basic & Clinical Immunology, 3rd Ed (1980) Lange Medical Publications, pp 187-190.

HLA cDNAs identified as potential probes may also be useful in making recombinant clones expressing human HLA antigens. In this connection, cDNA that encodes a given HLA antigen is inserted into an appropriate cloning vehicle (vector) and hosts are transformed with the vehicles. Transformants are isolated and those that produce the desired antigen are cloned. The antigen may be harvested from the clones by conventional methods. Such antigens may be useful for diagnostic purposes, for making anti-HLA antibodies, or for therapy to induce tolerance to HLA antigens.

The following examples further illustrate the various aspects of the invention. These examples are not intended to limit the invention in any manner.



Example 1: Preparation of Hybridization Probe for  
HLA-Dp34 (HLA-DR $\alpha$ )

Four 11-mer oligonucleotides were prepared based on the known NH<sub>2</sub>-terminal amino acid sequence (Glu, Phe, Tyr, Leu) of positions 11-14 of HLA-Dp34 antigen. The base sequences for the four oligonucleotides were as follows: (1) AGGTAAAATTC, (2) AGGTAGAATTC, (3) AGGTAAAACTC, and (4) AGGTAGAACTC. These sequences are all complementary to the codons for the indicated peptide sequence and were chosen to minimize degeneracy. The ambiguities are located at sequence positions 2, 3, 6, and 9. A G at positions 2 and 3 was chosen to minimize the destabilizing effect of potential mismatched bases (G is capable of forming a wobble pair with U).

Since the four oligonucleotides were complementary to codons for amino acids 11-14, oligonucleotide primed cDNA synthesis on HLA-Dp34 mRNA was expected to generate a product of about 150-200 nucleotides. This estimate was based on a leader sequence of ~75 nucleotides and assumes a 5' untranslated region of 75-125 nucleotides.

The specificities of the four 11-mers were compared by using them individually as primers in cDNA synthesis reactions using membrane-bound B cell mRNA, free B cell mRNA, and T cell mRNA as template. Only the AGGTAGAACTC oligonucleotide primed a cDNA band of ~175 nucleotides which was enriched in reactions on B cell membrane-bound mRNA template. The specificity of this 11-mer oligonucleotide was confirmed by extending the primer in a cDNA synthesis reaction in the presence of a single dideoxy triphosphate and three deoxy triphosphates, an approach which has proved successful in the isolation of the HLA-B7 cDNA clone

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(Sood, et al, supra). In the presence of dideoxy dATP, a minor cDNA band corresponding to a predicted 18-nucleotide primer extension product was observed. The additional seven nucleotides were determined by the wandering spot sequencing technique to be GGCCTGA. The following two additional nucleotides, AT, were inferred from the Ile codon, giving a nine nucleotide sequence that corresponded to the HLA-Dp34 antigen amino acids at positions 8, 9, and 10.

10 A 20-nucleotide fragment having the above determined sequence (AGGTAGAACTCGGCCTGAAT) was then synthesized by the triester method. The specificity of the 20-mer as a primer was examined in a cDNA synthesis reaction on poly(A<sup>+</sup>) mRNA from a B cell line. 15 A major cDNA band, 175 nucleotides long, was synthesized; the nucleotide sequence of the eluted band, corresponded to the expected sequence for HLA-Dp34.

The specificity of the 20-nucleotide fragment as a hybridization probe was analyzed on a 20 Northern blot of poly(A<sup>+</sup>) mRNA. A unique band, at 1200-1300 nucleotides resulted from probing B cell mRNA but not T cell mRNA with the <sup>32</sup>P-labeled 20-mer nucleotide probe. Membrane-bound mRNA was enriched for the mRNA which hybridized to the 20-nucleotide 25 probe.

An HLA-Dp34 cDNA clone was identified in a cDNA library with the above described 20-mer probe as follows. Membrane-bound RNA and free RNA was prepared, using phenol-chloroform extraction in the 30 presence of Vanadyl complexes, from the human lymphoblastoid B cell line, CA. Poly(A<sup>+</sup>) mRNA, isolated by affinity chromatography with Poly U-Sepharose, was translated in an in vitro rabbit reticulocyte system. The partition of specific mRNA's into the membrane-

bound and free fractions was monitored by 2D gel analysis of the  $^{35}\text{S}$ -labeled products of in vitro translation. A double-stranded cDNA library was prepared from the membrane-bound mRNA using reverse transcriptase, DNA Polymerase I, and S1 nuclease. Following tailing with dCTP using terminal transferase, the cDNA was inserted and ligated to preparations of the plasmid pBR322 which had been digested with Pst and tailed with dGTP.

- 10 Initial screening of the library was carried out as follows. Duplicate sets (~4,000 clones/set) of Grunstein-Hogness colony filters were prepared. One set was probed with  $^{32}\text{P}$  cDNA made from size fractionated mRNA from the B cell line, CA. Sucrose gradient
- 15 fractions were translated in an in vitro rabbit reticulocyte system and the  $^{35}\text{S}$ -labeled products analyzed by 2D gel electrophoresis to determine the appropriate fraction. The other set of filters was probed with  $^{32}\text{P}$  cDNA made from mRNA from the T cell line, Molt-4.
- 20 A subset of about 150 clones, derived from membrane-bound, B cell specific, 12-14s mRNA, was defined by this initial screening.

- Plasmid DNA was prepared from 25 pools, each consisting of 5 candidate cDNA clones and analyzed by
- 25 dot hybridization with the  $^{32}\text{P}$ -labeled 20-nucleotide probe. Pool 14 plasmid DNA hybridized specifically with the probe. Subsequently, the individual members of the pool were tested; cDNA sequences complementary to the hybridization probe were restricted to the
- 30 clone identified as 18C7.

In Northern blots, the  $^{32}\text{P}$ -labeled 18C7 nick translated probe hybridizes to a B cell mRNA of the same length (about 1200 to about 1300 nucleotides) as the band complementary to the 20-nucleotide probe. In

genomic blots with DNA from a hamster-human hybrid containing the human chromosomes 6 and 3, the 18C7 probe hybridizes to a unique restriction fragment absent in the hamster parent, mapping the 18C7 DNA sequences to chromosome 6.

A more precise mapping was possible using the cell line 6.3.6 which has a small deletion at a defined site on the short arm of one homologue of the chromosome 6 pair. This deletion variant fails to express the HLA-A, B, C and HLA-DR specificities associated with one chromosome 6 haplotype. In genomic blots (Fig 1 and Example 4 below), 18C7 hybridizes to two restriction fragments from the parent cell line, presumably from the two chromosome 6's. Only one fragment is observed in DNA from the deletion variant; the other fragment is presumably derived from the chromosome which has been deleted. This result maps DNA sequences complementary to the 18C7 clone to the chromosomal site defined by the 6.3.6 deletion.

The human HLA-D locus is homologous to the mouse I region. In genomic blots with DNA from mouse congenic lines, inbred lines which differ only at the I region, the 18C7 probe hybridized to a restriction fragment that was different with each congenic line. This result maps DNA sequences complementary to the 18C7 clone to the mouse I region and therefore to the human HLA-D locus.

The 18C7 clone was confirmed as being HLA-Dp34 (HLA-DR $\alpha$ ) by analyzing its DNA sequence by the Maxam-Gilbert technique (Methods in Enzymology (1980) 65:499-560) using the endonucleases PstI, HinfI, TaqI, Sau3A, AvaII, and BglI. The sequence for the coding strand of the HLA-Dp34 clone is given below (N = unidentified nucleotide).

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ATCATAGCTG TGCTGATGAG CGCTCAGGAA TCATGGGCTA TCAAAGAAGA  
 ACATGTGATC ATCCAGGCCG AGTTCTATCT GAATCCTGAC CAATCAGGCC  
 AGTTTATGTT TGACTTTGAT GGTGATGAGA TTTTCCATGT GGATATGGCA  
 AAGAAGGAGA CGGTCTGGCG GCTTGAAGAA TTTGGACGAT TTGCCAGCTT  
 5 TGAGGCTCAA GGTGCATTGG CCAACATAGC TGTGGACAAA GCCAACCTGG  
 AAATCATGAC AAAGCGCTCC AACTATACTC CGATCACCAA TGTACCTCCA  
 GAGGTAAGT TGCTCAGGAA CAGCCCTGTG GAACTGAGAG AGCCCAACGT  
 CCTCATCTGT TTCATCGACA AGTTCACCCC ACCAGTGGTC AATGTCACGT  
 GGCTTCGAAA TGGAAAACCT GTCACCACAG GAGTGTGAGA GACAGTCTTC  
 10 CTGCCCAGGG AAGACCACCT TTTCCGCAAG TTCCACTATC TCCCCTTCCT  
 GCCCTCAACT GAGGACGTTT ACGACTGCAG GGTGGAGCAC TGNGGCTTGG  
 ATGAGCCTCT TCTCAAGCAC TGGGAGTTTG ATGCTCCAAG CCCTCTCCCA  
 GAGACTACAG AGAACGTGGT GTGTGCCCTG GGCCTGACTG TGGGTCTGGT  
 GGGCATCATT ATTGGGACCA TCTTCATCAT CAAGGGAGTG CGCAAAGCA  
 15 ATGCAGCAGA ACGCAGGGGG CCTCTGTAAG GCACATGGAG GTGATGATGT  
 TTCTTAGAGA GAAGATCACT GAAGAACTT CTGCTTTAAT GACTTTACAA  
 AGCTGGCAAT ATTACAATCC TTGACCTCAG TGAAAGCAGT CATCTTCAGC  
 GTTTTCCAGC CCTATAGCCA CCCCAAGTGT GGTTATGCCT CCTCGATTGC  
 TCCGTACTCT AACATCTAGC TGGCTTCCCT GTCTATTGCC TTTTCTGTGA  
 20 TCTATTTTCC TCTATTTTCT ATCATTTTAT TATCACCATG CAATGCCTCT  
 GGAATAAAAC ATACAGGAGT CTGTCTCTGC TATGGAATGC CCCATGGGGC  
 TCTCTTGTGT ACTTATTGTT TAAGGTTTCC TCAAAGTGTG ATTTTCTG

A  $^{32}\text{P}$ -labeled HLA-Dp34 probe was made from the clone by nick translation.

25 Example 2: Preparation of Hybridization Probe for Ia Like Class II HLA p29G8

The HLA-DR $\alpha$  related or human Ia like clone, p29G8, was identified by screening the cDNA library of Example 1 with the nick-translated HLA-Dp34 (DR $\alpha$ )  
 30 probe under hybridization conditions of reduced stringency to allow detection of related but distinct DNA

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sequences. The hybridization conditions were as follows.

Hybridize in 50% formamide, 5 x SSPE (1 x SSPE = 0.18 M NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM Na<sub>2</sub>EDTA, pH 7.0), 0.1% sodium dodecyl sulfate (SDS), 5 x Denhardt's (5 x Denhardt's = 0.1% w/v each bovine serum albumin, Ficoll, polyvinyl pyrrolidone), 200 µg/ml sheared denatured salmon sperm DNA, at 37°C for 24 h with 1 x 10<sup>6</sup> cpm <sup>32</sup>P labeled HLA-Dp34 probe (2 x 10<sup>8</sup> cpm/µg, labeled by nick translation). Wash filters 3 x 15 min at room temperature in 5 x SSPE, 0.1% SDS.

Fifteen HLA-DRα related cDNA clones were identified. Among them was a clone designated p29G8. Under conditions of high stringency (wash at 0.1 x SSPE, 65°C), p29G8 hybridizes strongly only to itself. The p29G8 clone (minus strand) was partially sequenced using the Maxam-Gilbert procedure. Four fragments, designated A1, A2, B1, and B2, were obtained by digesting the p29G8 clone. The clone was first digested with PstI to yield a ~400 bp fragment and a ~600 bp fragment. The larger fragment was cut with Sau3A to give fragment A1 or with MspI to give fragment A2. The smaller fragment was cut with HaeIII to give fragment B1 or with Sau3A to give fragment B2. The sequences of these fragments are reported below (N = unidentified nucleotide).

Fragment A1

TNTGAACNCCAGCTGCCCTACAACTCCATCTCAGCTTTCTTCTCACTTCATG  
TNAAACTACTCCAGTGGCTGACTNAATTGCTGACCCTTCAAGCTCTGTCCTTA  
TCCATTACCTCAAAGCAGTCATTCCTTAGTNAAGTTTCCAAC

## Fragment A2

CACGGGAGNCCCAAGAGCCAACCAGACGCCTGAGACAACGGAGACTGTGCTCTG  
 TGCCCTGGGCCTGGTGCTGGGCCTAGTCGGCATCATCGTGGGCACCGTCCTNAT  
 CATAAAGTCTCTGCGTTCTGGCCATGACCCCC

## 5 Fragment B1

CACATTGACGAGTTCTTCCCACCAGTCCTCAANGTCACGTGGGCCGCGCAACGG  
 GGAGCTGGTCACTGAGGG

## Fragment B2

AAGGAGACCGTCTGGCATCTGGAGGAGTTTGGCCAAGCCTTTTCCCTTTGAGGC  
 10 TCAGGGCGGGCTGGCTAACATTGCTATATTGAACAACAACCTTGAAACCTTGA

The partial sequence is distinct from the sequence for  
 HLA-Dp34 (DR $\alpha$ ) and differs from the recently published  
 nucleotide sequence for the HLA-DC1 clone (Auffrey, et  
 al, (1982) PNAS Vol 79:6637-6341) both in sequence and  
 15 length of the 3' untranslated region. In genomic  
 Southern blots, the p29G8 probe hybridizes to genomic  
 restriction fragments distinct from those which hybri-  
 dize to the HLA-Dp34 (DR $\alpha$ ) probe in DNA from an HLA  
 hemizygous cell line (6.3.6). This observation indi-  
 20 cates that p29G8 represents a different (new) locus  
 and not simply another allele. The genomic blot pat-  
 tern with DNA from the cell line T5-1 and its HLA  
 hemizygous derivative 6.3.6 indicates that the p29G8  
 locus maps within the HLA region.

25 Example 3: Characterization of Other Class II HLA  
cDNA Clones

DR $\alpha$ 

As indicated in Example 2, fourteen other  
 DR $\alpha$ -related cDNA clones were identified. Four of  
 30 these clones are very strongly homologous to the HLA-  
 Dp34 (DR $\alpha$ ) clone and are probably identical or allelic  
 copies. Thus, ten clones represent candidates for new  
 HLA-DR $\alpha$  related loci. These ten clones are listed  
 below.

-23-

	p14A9	p11G7
	p17D8	p36A1
	p22G12	p51H2
	p40G4	p25A6
5	p6B3	p36F7

Clone p14A9 hybridizes to genomic restriction fragments distinct from those that hybridize to the HLA-Dp34 (DR $\alpha$ ) and p29G8 probes. Thus, p14A9 represents a third DR $\alpha$  related locus. By comparing the pattern of cross-hybridization of these HLA-DR $\alpha$  related clones, it was estimated that these 10 HLA-DR $\alpha$  related clones represent a minimum of two and a maximum of 10 different HLA-DR $\alpha$  related loci.

#### DR $\beta$

15 An 18-mer having the base sequence CCCTGTCTCGCGCACGCA was prepared for use in screening a cDNA library of HLA-DR $\beta$  clones. The sequence of the 18-mer was based on the published amino acid sequence for the conserved amino acids 20-25 of the HLA-DR $\beta$  chain. The specific 18-nucleotide sequence was chosen from the published sequence of an HLA-DR $\beta$  cDNA clone from the Raji cell line (Wiman et al (1982) PNAS 79:1703-1707). The specificity of the kinased 18-mer probe was tested by hybridization to an RNA blot. The probe hybridized to an RNA species about 1100-1300 nucleotides long present in B cell RNA and absent from T cell RNA, as expected for the HLA-DR $\beta$  mRNA. Hybridization conditions were for 36 h at 37°C in 4 x SSPE with 5 x Denhardt's solution, 0.2 mM ethylene diamine-tetraacetic acid (EDTA), and 0.1% SDS. Filters were washed in 2 x SSPE, 0.1% SDS at room temperature.



The 18-mer probe was hybridized to a cDNA bank derived from mRNA from the  $\beta$  lymphoblastoid cell line, LG2. The cDNA bank was constructed by inserting the duplex cDNA ligated to EcoRI linkers into the EcoRI site of the  $\lambda$ gt 10 vector. cDNA inserts from eight 18-mer reactive  $\lambda$  cDNA clones were isolated and subcloned into the EcoRI site of the plasmid vector, pBR328 (Soberon, X., et al, Gene (1980) 9:287-305). The clones designated DR $\beta$ -4 and DR $\beta$ -8 hybridize to sequences in the HLA region using the genomic blotting technique with the 6.3.6 HLA hemizygous deletion variant described in Example 1. The products of in vitro translation of mRNA which hybridize to DR $\beta$  chains have the electrophoretic mobility expected of HLA-DR $\beta$  chains. In oocytes, the translation products of specifically hybridizing mRNAs associate with the translation products of HLA-DR $\alpha$  and HLA-DR $\gamma$  mRNAs. Thus, by a variety of criteria, these clones encode HLA-DR $\beta$  or DR $\beta$  like proteins. The genomic blot patterns obtained with the HLA-DR $\beta$ -4 and HLA-DR $\beta$ -8 probes are different indicating these clones represent different loci.

Example 4: Determination of HLA-DR $\alpha$  Restriction  
Fragment Length Polymorphism Using BglII and HLA-Dp34  
Hybridization Probe.

Digestion of DNA with BglII

Samples of DNA were obtained from nucleated cells of five unrelated individuals and four children of two of the individuals. Five to ten  $\mu$ g of each DNA sample were digested for 1-2 hr at 37°C with 4(15') units of BglII per  $\mu$ g of DNA. The buffer was 60 mM NaCl, 6 mM Tris HCl (pH 7.5), 6 mM MgCl<sub>2</sub>, 6 mM 2-mercaptoethanol.

Genomic Blotting of Restriction Digests with HLA-Dp34  
Probe

Between five and ten  $\mu\text{g}$  of each restriction enzyme digested DNA was fractionated according to size by electrophoresis in a 0.6% agarose gel for approximately 500 V-hr. Electrophoresis buffer was 40.0 mM Tris, 2.5 mM EDTA, 25.0 mM acetate, pH 8.2. After electrophoresis the DNA was stained with 0.5  $\mu\text{g}/\text{ml}$  ethidium bromide and the gel was photographed. The DNA in the gel was depurinated with a 15' wash in 75 mM HCl at room temperature. DNA was denatured with 2 successive 15' washes in 0.5 M NaOH + 1.5 M NaCl at room temperature. DNA was neutralized with 2 successive 15' washes in 1.5 M Tris-Cl pH 7.4 + 3.0 M NaCl at room temperature. DNA was transferred from the gel to nitrocellulose (0.45 micron pore size) by blotting with 20 x SSPE (20 x SSPE = 3.6 M NaCl, 200.0 mM phosphate, 20.0 mM  $\text{Na}_2\text{EDTA}$ , pH 7.0) for 3 hr at room temperature. DNA was bound to the nitrocellulose by baking at 80°C for 2 hr in a vacuum oven.

The nitrocellulose filter was placed in a heat sealable plastic bag and was prehybridized for 8 hr at 42°C in a solution composed of 50% formamide, 5 x SSPE, 200  $\mu\text{g}/\text{ml}$  sheared denatured salmon sperm DNA, 0.1% SDS, and 2 x Denhardt's solution. After 8 hr, the prehybridization solution was removed from the bag and replaced with a solution composed of 50% formamide, 5 x SSPE, 100  $\mu\text{g}/\text{ml}$  sheared denatured salmon sperm DNA, 0.1% SDS, 2 x Denhardt's solution, 10% sodium dextran sulphate, and  $1-5 \times 10^6$  cpm denatured  $^{32}\text{P}$  labelled HLA-Dp34 probe. The probe was labeled using the nick translation reaction of Rigby, et al, supra, to specific activities of

5 x 10<sup>8</sup> -1 x 10<sup>9</sup> cpm/μg. The bag was resealed and the nitrocellulose filter was hybridized with the probe for 18-24 hr at 42°C.

The nitrocellulose filter was removed from the bag and washed in 4 successive changes (15' each) of 2 x SSPE, 0.17% SDS at room temperature. The filter was then washed in 4 successive changes (15' each) of 0.1 x SSPE, 0.1% SDS at 50°C.

The filter was air dried, covered with Saran Wrap and autoradiographed with Kodak XAR-5 film with an intensifying screen for 18-72 hr at -80°C. Fig 1 is a copy of the resulting autoradiograph.

#### Discussion of Autoradiograph

The autoradiograph shows that BglIII produced three different restriction fragments in the tests: 3.8, 4.2, and 4.5 kb in length. This clearly evidences that the HLA-Dp34 locus is polymorphic and that there are at least three alleles of this gene.

Lane 1 of the autoradiograph is a blot of the cell line CA. Lanes 3 and 2 of the autoradiograph are blots of the cell line 6.3.6 and its parent T5-1, respectively. These are the blots discussed in Example 1 that map the HLA-Dp34 gene to chromosome 6 at the site defined by the 6.3.6 deletion.

Lanes 4 and 5 are blots of the mother and father of a family and lanes 6-9 are blots of the children of those parents. Lane 4 is the father's blot and his haplotype is designated A/B. Both chromosome A and chromosome B have the same restriction fragment (4.2 kb). Lane 5 is the mother's blot and her haplotype is designated C/D. Each chromosome C and D has a different restriction fragment (4.2 kb and 4.5 kb, respectively). In the offspring of these par-

ents the maternal 4.2 kb genomic fragment segregates with the serologically defined D haplotype.

Example 5: HLA-Dp34 Typing Based on RFLP Using HLA-Dp34 Hybridization Probe and the Restriction

5 Endonuclease BglII

A sample of peripheral blood is obtained from an individual and the HLA DNA is extracted therefrom using the methods described in Example 4. The DNA is digested using BglII and a genomic blot of the  
10 digest is made using the HLA-Dp34 as described in Example 4. The restriction fragment pattern of the resulting autoradiograph is compared to the restriction fragment patterns described in Example 4 (Fig. 1) for HLA-Dp34 to determine the individual's HLA-Dp34  
15 type.

Example 6: Restriction Fragment Length Polymorphisms Detected with EcoRV and HLA-Dp34 (DR $\alpha$ ) Probe

Ten  $\mu$ g samples of DNA were obtained from three individuals and digested for 1-4 hr at 37°C with  
20 80 units of EcoRV. The buffer was 0.15 M NaCl, 6 mM Tris HCl (pH 7.9), 6 mM MgCl<sub>2</sub>, 6 mM 2-mercaptoethanol. Genomic blotting and hybridization of restriction digested DNAs were carried out as in Example 4. An autoradiograph was prepared of the genomic blotting  
25 patterns as in Example 4. Fig 2 is a copy of the resulting autoradiograph.

As seen in Fig 2, all three individuals possess a 986 bp and a 6.8 kb EcoRV fragment (nonpolymorphic fragments). In addition, every individual pos-  
30 sesses either a 9.2 kb or a 13.0 kb EcoRV fragment, or both (polymorphic fragments). Fig 3 is a map diagram-

ming the location of the polymorphic restriction sites for BglIII (Example 4) and EcoRV detected with the HLA-Dp34 probe.

5 Example 7: Determination of Restriction Fragment Length Polymorphism in Class II Locus Using BglIII and Hybridization Probe p29G8

Ten  $\mu$ g samples of DNA from five individuals of one family and five individuals of a second family were digested with BglIII using the procedure of

10 Example 4. The digests were subjected to electrophoresis, genomic blotting and hybridization by the procedure of Example 4 except that the nick translated p29G8 probe of Example 2 was used instead of the HLA-Dp34 probe. Fig 4 is an autoradiograph of the resulting genomic blotting patterns. Lanes 1-5 are the

15 patterns for the five individuals of the first family and Lanes 6-10 are the patterns of the five individuals of the second family. As seen in Fig 4 three types ("alleles") were observed in the samples characterized by 2.2 kb, 2.4 kb, and 4.4 kb fragments. In

20 the HLA typed families of this example the polymorphic BglIII fragments segregate with serologically defined parental haplotypes.

25 Example 8: Use of HLA-B7 Hybridization Probe to Evaluate HLA Restriction Fragment Length Polymorphisms in Human Pedigrees

An HLA-B7 cDNA clone was obtained from Sherman M. Weissman, Dept of Human Genetics, Yale University School of Medicine. This clone is

30 described in Sood, et al, supra.

DNA samples from the father, mother, and four children of an HLA typed family and in two

instances DNA from one or two individuals (designated X and Y) unrelated to that family were digested according to Example 4 with either HindIII, PvuII, or BamHI. Genomic blots of the digests were made as in  
5 Example 4 using the  $^{32}\text{P}$  nick translated HLA-B7 clone as a hybridization probe. Figs 5, 6 and 7 are copies of the resulting autoradiographs. The father's haplotype is designated A/D and the mother's haplotype is designated E/F. These autoradiographs indicate that  
10 the probe is a multilocus probe that hybridizes to more than one HLA locus. Nonetheless, several polymorphic bands, segregating in the pedigree, are present for each enzyme used. Moreover, the bands segregate with the serologically defined HLA loci so  
15 that given fragments may be assigned to an individual chromosome.

Exclusionary paternity determinations may be made using autoradiographs such as Figs 5, 6 and 7. An exclusionary pattern would involve a restriction  
20 fragment pattern which could not be inherited from the mother and the alleged father. Such a pattern would be one in which the child has a fragment that neither the mother nor the alleged father has. Positive paternity determinations using RFLPs will depend upon the  
25 frequency of the RFLPs in the general population. In such determinations one calculates the probability that the putative father contributed the RFLP that is observed and compares it with the probability that any random male would contribute the RFLP to the child.

Example 9: Use of BglIII and HLA-Dp34 (DR $\alpha$ ) Probe to  
Evaluate Linkage and Association Between HLA-DR $\alpha$  and  
Insulin Dependent Diabetes Mellitus (IDDM) in Six  
Families

5           Ten  $\mu$ g samples of DNA from the individuals  
of six different families having histories of IDDM  
were digested with BglIII, blotted, and hybridized with  
the HLA-Dp34 probe by the procedure of Example 4.  
10        Autoradiographs of the resulting genomic blotting  
patterns were made.

          Fig 8 is an autoradiograph of eight individ-  
uals of one of the families. The father's pattern  
appears in Lane 1, the mother's in Lane 2 and six  
children in Lanes 3-8. The father has the 4.2 kb  
15       fragment (derived from chromosome 6 haplotype A) and  
the 3.8 kb fragment from haplotype B. The mother has  
the 4.2 kb fragment (haplotype C) and the 4.5 kb frag-  
ment (haplotype D). The three affected (IDDM) chil-  
20       dren have either two copies of the 4.2 kb fragment  
(haplotype A/C, Lanes 3 and 5) or one copy of the  
4.2 kb fragments (haplotype A) and one copy of the  
4.5 kb fragment (haplotype D) in Lane 4. One unaffec-  
ted child (Lane 7) has two copies of the 4.2 kb frag-  
ment (haplotype A/C). The two unaffected children  
25       (Lanes 6 and 8) both have the 3.8 kb fragment (haplo-  
type B) and the 4.5 kb fragment (haplotype D). Thus,  
the 4.2 kb fragment is linked in this family to a  
disease susceptibility gene for IDDM. Three of the  
four children with the 4.2 kb fragment exhibit IDDM,  
30       indicating an incomplete penetrance (genetic predis-  
position) of the disease allele.

          In four of the other five families tested  
the segregation pattern was consistent with linkage of  
the 4.2 kb BglIII fragment to the IDDM disease suscep-  
35       tibility allele.

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Fig 9 is an autoradiograph of five individuals of the sixth family. The father's pattern appears in Lane 5, the mother's in Lane 4 and the three children in Lanes 1-3. In this family, disease susceptibility appeared to be linked to the 4.5 kb BglII fragment. Here, the affected father has a 4.5 kb fragment (haplotype A) and 4.2 kb fragment (haplotype B) and the mother has a 4.5 kb fragment (haplotype D) and a 3.8 kb fragment (Haplotype C). Of the three children, only one has IDDM (Lane 3) and he inherited the 4.5 kb fragment from the father and the 3.8 kb fragment from the mother. Therefore, in this family the 4.5 kb BglII fragment is linked to the disease susceptibility allele on the paternal haplotype A.

In summary, five of the six probands in this set of diabetes families had at least one copy of the 4.2 kb BglII fragment. In a sample of unrelated control individuals, 4/16 individual DNA samples had the 4.2 kb fragment, suggesting an increased relative risk (RR) for IDDM associated with the 4.2 kb fragment due, presumably, to linkage disequilibrium between the polymorphic BglII site and a linked IDDM susceptibility allele.

$$RR \text{ (relative risk)} = \frac{(.834) \times (.75)}{(.25) (.167)} = 14$$

By comparison, the relative risk for the serologically defined HLA-DR3 = 3.3 and for HLA-DR4 = 6.4. By using more restriction endonucleases and more probes, it should be possible to generate a significantly higher value than the one indicated by these data with the HLA-DR $\alpha$  probe and BglII.



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Claims

1. An HLA typing method based on HLA DNA restriction fragment length polymorphism characterized by the following steps:

5 (a) digesting HLA DNA from an individual with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA;

(b) subjecting the digest of (a) to genomic blotting or solution hybridization - resolution using  
10 a labeled cDNA probe that is complementary to an HLA DNA locus sequence involved in the polymorphism; and

(c) comparing the genomic blotting or resolution pattern obtained in (b) with a standard genomic blotting or resolution pattern for said HLA DNA  
15 sequence obtained using said restriction endonuclease and an equivalent labeled cDNA probe.

2. The method of Claim 1 further characterized in that the probe is specific to a single HLA locus and the restriction endonuclease is one that  
20 produces a polymorphic digestion pattern associated with the DNA of said locus.

3. The method of Claim 2 further characterized in that said locus is a Class II locus.

4. The method of Claim 3 further characterized in that the locus is the DR $\alpha$  locus, a DR $\alpha$  related locus, or the DR $\beta$  locus.  
25

5. The method of Claim 3 further characterized in that:

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- (i) the locus is the DR $\alpha$  locus and the restriction endonuclease is BglII or EcoRV, or
- (ii) the locus is a DR $\alpha$  related locus and the restriction endonuclease is BglII, or
- (iii) the locus is the DR $\beta$  locus or a DR $\beta$  related locus and the restriction endonuclease is EcoRI or BglII.

6. A method for determining the paternity of an individual based on HLA DNA restriction fragment length polymorphism characterized by the following steps:

(a) digesting HLA DNA of the mother of the individual, the suspected father of the individual, and the individual with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA;

(b) subjecting each of the digests of (a) to genomic blotting or solution hybridization - resolution using a labeled cDNA probe that is complementary to an HLA DNA sequence involved in the polymorphism; and

(c) comparing the genomic blotting or resolution patterns obtained in (b) to determine correspondence between the individual's pattern and the mother's pattern and suspected father's pattern and thereby determining whether the suspected father is the actual father of the individual.

7. A method for determining transplant or transfusion compatibility based on HLA DNA restriction fragment length polymorphism characterized by the following steps:

(a) digesting HLA DNA of the transplant or transfusion donor and the transplant or transfusion host with a restriction endonuclease that produces a polymorphic digestion pattern with HLA DNA;

5 (b) subjecting each of the digests of (a) to genomic blotting or solution hybridization - resolution using a labeled cDNA probe that is complementary to an HLA DNA sequence involved in the polymorphism; and

10 (c) comparing the genomic blotting or resolution patterns obtained in (b) to determine correspondence therebetween and thereby determining whether the transplant or transfusion donor's HLA is compatible with the transplant or transfusion host's HLA.

15 8. A method of determining whether an individual is susceptible to or has a disease characterized by the following steps:

(a) digesting HLA DNA from the individual with a restriction endonuclease that produces a polymorphic digestion pattern of an HLA locus that is  
20 associated with the disease;

(b) subjecting the digest of (a) to genomic blotting or solution hybridization - resolution using a labeled cDNA probe that is complementary to the DNA  
25 sequence of said HLA locus;

(c) comparing the genomic blotting or resolution pattern obtained in (b) with a standard pattern of an individual having said disease obtained using said restriction endonuclease and an equivalent  
30 labeled cDNA probe.

9. The method of claim 8 wherein the disease is insulin dependent diabetes mellitus.

10. The method of claim 8 wherein said HLA locus is a Class II locus.

11. An HLA cDNA probe characterized in that the probe is specific to a single HLA locus and comprises a labeled DNA sequence that is complementary to the DNA sequence at said locus.

12. The probe of Claim 11 further characterized in that the locus is a Class II locus.

13. The probe of Claim 12 further characterized in that the locus is the DR $\alpha$  locus, a DR $\alpha$  related locus, the DR $\beta$  locus, or a DR $\beta$  related locus.

14. The probe of claim 12 further characterized in that the locus is the DR $\alpha$  locus and the nucleotide sequence of the probe is

15 ATCATAGCTG TGCTGATGAG CGCTCAGGAA TCATGGGCTA TCAAAGAAGA  
ACATGTGATC ATCCAGGCCG AGTTCTATCT GAATCCTGAC CAATCAGGCG  
AGTTTATGTT TGA CTTTGAT GGTGATGAGA TTTTCCATGT GGATATGGCA  
AAGAAGGAGA CGGTCTGGCG GCTTGAAGAA TTTGGACGAT TTGCCAGCTT  
TGAGGCTCAA GGTGCATTGG CCAACATAGC TGTGGACAAA GCCAACCTGG  
20 AAATCATGAC AAAGCGCTCC AACTATACTC CGATCACCAA TGTACCTCCA  
GAGGTAAC TGCTCACGAA CAGCCCTGTG GAACTGAGAG AGCCCAACGT  
CCTCATCTGT TTCATCGACA AGTTCACCCC ACCAGTGGTC AATGTCACGT  
GGCTTCGAAA TGGAAAACCT GTCACCACAG GAGTGTGAGA GACAGTCTTC  
CTGCCCAGGG AAGACCACCT TTTCCGCAAG TTCCACTATC TCCCCTTCCT  
25 GCCCTCAACT GAGGACGTTT ACGACTGCAG GGTGGAGCAC TGNGGCTTGG  
ATGAGCCTCT TCTCAAGCAC TGGGAGTTTG ATGCTCCAAG CCCTCTCCCA  
GAGACTACAG AGAACGTGGT GTGTGCCCTG GGCCTGACTG TGGGTCTGGT  
GGGCATCATT ATTGGGACCA TCTTCATCAT CAAGGGAGTG CGCAAAGCA  
ATGCAGCAGA ACGCAGGGGG CCTCTGTAAG GCACATGGAG GTGATGATGT

TTCTTAGAGA GAAGATCACT GAAGAACTT CTGCTTTAAT GACCTTACAA  
 AGCTGGCAAT ATTACAATCC TTGACCTCAG TGAAAGCAGT CATCTTCAGC  
 GTTTTCCAGC CCTATAGCCA CCCCAGTGT GGTATGCCT CCTCGATTGC  
 TCCGTACTCT AACATCTAGC TGGCTTCCCT GTCTATTGCC TTTTCCTGTA  
 5 TCTATTTTCC TCTATTTTCT ATCATTTTAT TATCACCATG CAATGCCTCT  
 GGAATAAAAC ATACAGGAGT CTGTCTCTGC TATGGAATGC CCCATGGGGC  
 TCTCTTGTGT ACTTATTGTT TAAGGTTTCC TCAAACGTG ATTCTTCTG.

15. The probe of claim 12 wherein the locus  
 is DR $\alpha$  related and the probe contains the following  
 10 subsequences

(i)

TNTGAACNCCAGCTGCCCTACAACTCCATCTCAGCTTTTCTTCTCACTTCATG  
 TNAAAATACTCCAGTGGCTGACTNAATTGCTGACCCTTCAAGCTCTGTCCTTA  
 TCCATTACCTCAAAGCAGTCATTCCTTAGTNAAGTTTCCAAC,

15 (ii)

CACGGGAGNCCCAAGAGCCAACCAGACGCCTGAGACAACGGAGACTGTGCTCTG  
 TGCCCTGGGCCTGGTGCTGGGCCTAGTCGGCATCATCGTGGGCACCGTCCTNAT  
 CATAAAGTCTCTGCGTTCTGGCCATGACCCCC,

(iii)

20 CACATTGACGAGTTCTTCCCACCAGTCCTCAANGTCACGTGGGCCGCGCAACGG  
 GGAGCTGGTCACTGAGGG, and

(iv)

AAGGAGACCGTCTGGCATCTGGAGGAGTTTGGCCAAGCCTTTTCCCTTTGAGGC  
 TCAGGGCGGGCTGGCTAACATTGCTATATTGAACAACAACCTTGAAACCTTGA.

25 16. An HLA cDNA sequence characterized in  
 that the sequence is complementary to a single  
 HLA locus.

17. The cDNA sequence of claim 16 further  
 characterized in that the HLA locus is a Class II  
 30 locus.

18. The cDNA sequence of claim 17 further characterized in that the HLA locus is the DR $\alpha$  locus and the sequence is

```
ATCATAGCTG TGCTGATGAG CGCTCAGGAA TCATGGGCTA TCAAAGAAGA
5 ACATGTGATC ATCCAGGCCG AGTTCTATCT GAATCCTGAC CAATCAGGCG
  AGTTTATGTT TGACTTTGAT GGTGATGAGA TTTTCCATGT GGATATGGCA
  AAGAAGGAGA CGGTCTGGCG GCTTGAAGAA TTTGGACGAT TTGCCAGCTT
  TGAGGCTCAA GGTGCATTGG CCAACATAGC TGTGGACAAA GCCAACCTGG
  AAATCATGAC AAAGCGCTCC AACTATACTC CGATCACCAA TGTACCTCCA
10 GAGGTAAGTG TGCTCACGAA CAGCCCTGTG GAACTGAGAG AGCCCAACGT
  CCTCATCTGT TTCATCGACA AGTTCACCCC ACCAGTGGTC AATGTCACGT
  GGCTTCGAAA TGGAAAACCT GTCACCACAG GAGTGTGAGA GACAGTCTTC
  CTGCCCAGGG AAGACCACCT TTTCCGCAAG TTCCACTATC TCCCTTCCT
  GCCCTCAACT GAGGACGTTT ACGACTGCAG GGTGGAGCAC TGNGGCTTGG
15 ATGAGCCTCT TCTCAAGCAC TGGGAGTTTG ATGCTCCAAG CCCTCTCCCA
  GAGACTACAG AGAACGTGGT GTGTGCCCTG GGCCTGACTG TGGGTCTGGT
  GGGCATCATT ATTGGGACCA TCTTCATCAT CAAGGGAGTG CGCAAAAAGCA
  ATGCAGCAGA ACGCAGGGGG CCTCTGTAAG GCACATGGAG GTGATGATGT
  TTCTTAGAGA GAAGATCACT GAAGAACTT CTGCTTTAAT GACTTTACAA
20 AGCTGGCAAT ATTACAATCC TTGACCTCAG TGAAAGCAGT CATCTTCAGC
  GTTTTCCAGC CCTATAGCCA CCCCAAGTGT GGTTATGCCT CCTCGATTGC
  TCCGTACTCT AACATCTAGC TGGCTTCCCT GTCTATTGCC TTTTCCTGTA
  TCTATTTTCC TCTATTTCCCT ATCATTTTAT TATCACCATG CAATGCCTCT
  GGAATAAAAC ATACAGGAGT CTGTCTCTGC TATGGAATGC CCCATGGGGC
25 TCTCTTGTGT ACTTATTGTT TAAGGTTTCC TCAAAGTGT ATTTTCTG.
```

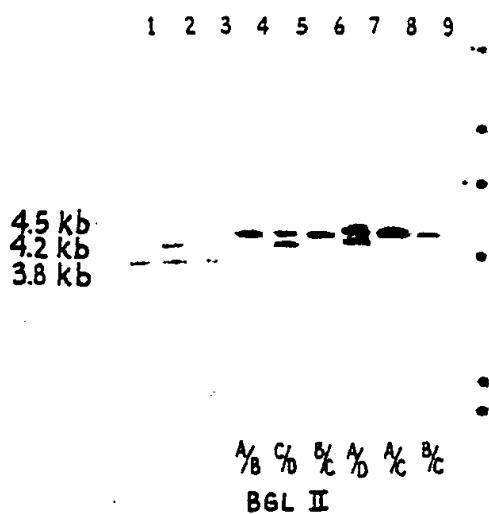


FIG. 1

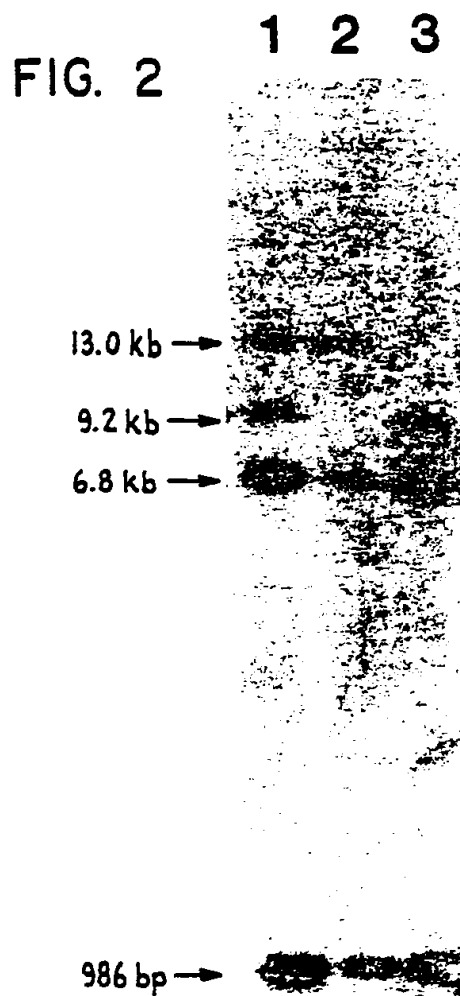


FIG. 2





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3/6

EP-56 901

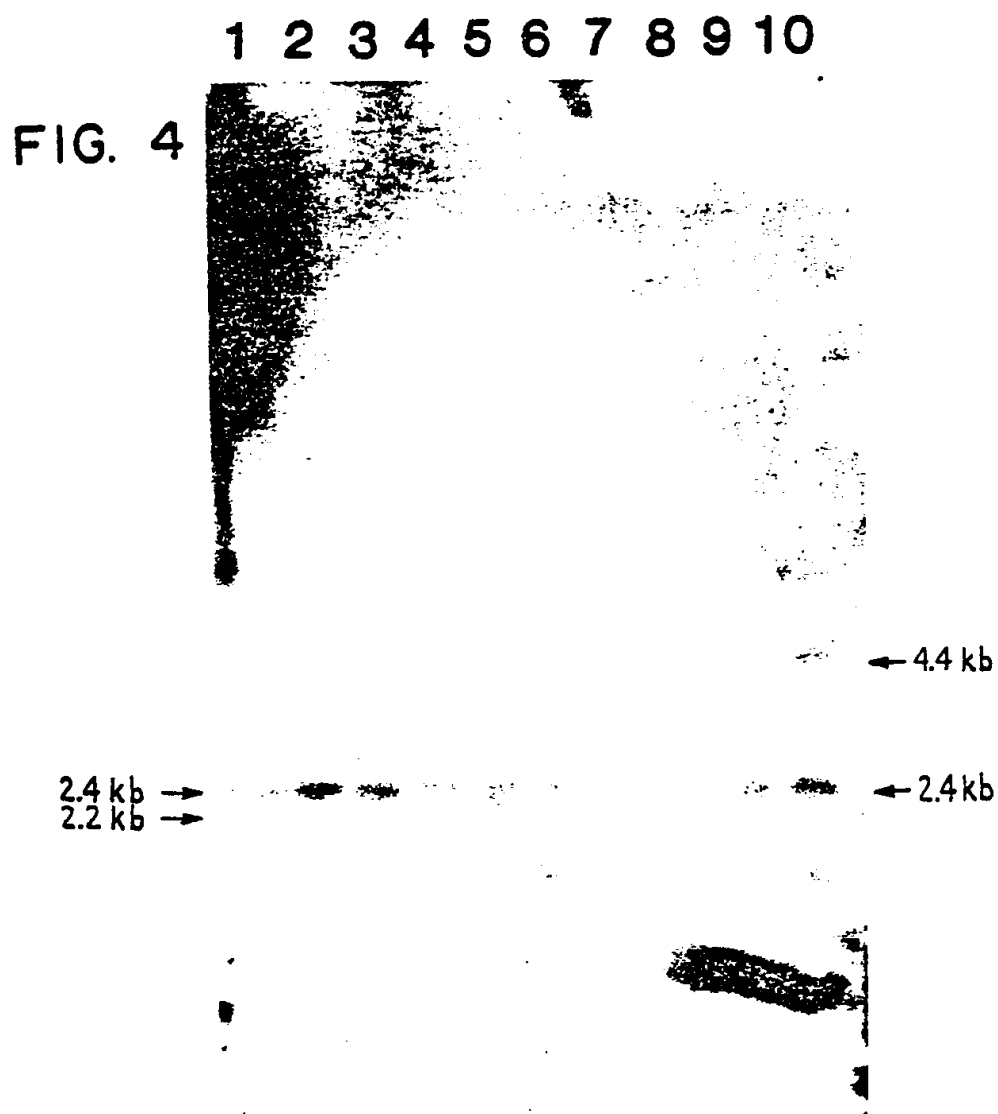


FIG. 5  
HIND III

A/D  
E/F  
D/E  
D/F  
D/E  
A/E

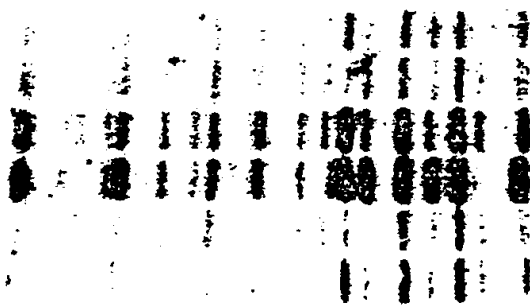


FATHER  
MOTHER  
CHILD 1  
CHILD 2  
CHILD 3  
CHILD 4  
INDIVIDUAL X  
INDIVIDUAL Y

FIG. 6  
Pvu II



A/D  
E/F  
D/E  
D/F  
A/E  
D/E



FATHER  
MOTHER  
CHILD 1  
CHILD 2  
CHILD 4  
CHILD 3

FIG. 7  
BAM HI

A/D  
E/F  
D/E  
D/F  
D/E  
A/E



FATHER  
MOTHER  
CHILD 1  
CHILD 2  
CHILD 3  
CHILD 4  
INDIVIDUAL X

